Fuchs et al

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Immunologically Active Proteines From Borrelia Burgdorferi, Related Test Kits and Vaccine

In the German Republic, lyme-borreliosis is the most common of tick-carried infectious diseases in humans. In contrast to early summer meningeoencephalitis (FSME), which is likewise carried by ticks, lyme-borreliosis is not limited to small endemic regions, but shows up in all of the States of the German Republic. The infection of the main vector in Europe, Ixodes ricinus, with the agent of lyme-borreliosis, the spirochete Borrelia burgdorferi, has reached in the South about 20% in the adult form, about 10% in the nymph form and about 1% in the larval form. The main vector in the U.S.A., Ixodes dammini, can be up to 100% infected with Borrelia.

B. burgdorferi belongs to the family of spirochetes. Spirochetes are helical bacteria of from 8-30 μm in length. They consist of an outer envelope, endoflagella in the periplasma, and the protoplasmic cylinder. The protoplasmic cylinder is a complex of cytoplasma, inner cell membrane and peptidoglycan. Among the human-pathogenic representatives of the spirochetes are found, in addition to B. burgdorferi, the relapse fever borrelia (for example, B. recurrentis), the agent of syphilis (Treponema (T.) pallidum) and the leptospira. Because of the close immunological relationship of the agents, cross reactions are a problem for serological evidence of antibodies for syphilis and lyme-borreliosis, with the tests that have been available thus far.

Infection with B. burgdorferi leads to a complex illness portrait that, as with syphilis, can be divided into three

different stages. The most important manifestations are:

Early phase:	Stage I	erythema migrans
		lymphocytar meningeoradiculitis Bannwarth (LMR)
		borrelia-lymphocytoma
Later Phase:	Stage III	lyme-arthritis
		acrodermatitis chronica atrophicans (ACA)
		chronic borrelia-encephalomyelitis

Rarer clinical manifestations are: carditis, myositis, iritis and panophthalmitis. Diaplacentar transfer of the agent is possible, however only a few cases of congenital lyme borreliosis have been documented up to now. The different stages can show either individually or in combination. Infection with B. burgdorferi can take place subclinically. Epidemiological studies of 375 clinically authenticated cases have shown certain anomalies in the age- and sex-distribution for the different clinical manifestations. For example, patients with erythema migrans occurred most frequently in the age group 30-60 years old. Neurological manifestations showed two age-spikes: the first in children and youths up to 20 years old, the second in the range 40 to 70 years. Lyme-arthritis was the most frequent in the age span 30-60 years. In no cases were patients with ACA younger than 30 years old. ACA was clearly more frequent in women than in men. Serological investigations using the immuno-fluorescence test showed preponderantly positive IgM in patients with erythema, and

preponderantly positive IgG in patients with neurological manifestations. For late-manifesting ACA and lyme-arthritis, the IgG titer was increased as a rule, and IgM antibodies were evident only in exceptional cases.

For the diagnostic process, one can use either the evidence of the agent or the evidence of the antibodies. Searching for evidence of the agent in the patient (skin biopsies, liquor, tapping) is recommended primarily for the early stage (erythema migrans), in which antibody evidence is usually negative. In any event, a complex nutrient medium is necessary for the culture of B. burgdorferi (Preac-Mursic, V.; Wilske, B.; Schierz, G. (1986): European Borreliae burgdorferi isolated from humans and ticks - culture conditions and antibiotic susceptibility. Zbl.Bakt.Hyg.A 163, 112-118), and for this reasons the culture requires special effort. In order to isolate the agent, a period up to five weeks is necessary. B. Burgdorferi can be isolated from skin samples in 50-70% of the cases with skin manifestations and in 3-5% of cases with neuroborreliosis (Preac-Mursic, V.; unpublished results).

The demonstration of antibodies (IgM, IgG) is carried out using serum, and in the case of neurological manifestations also using liquor. The serological finding is dependent upon the stage of the illness, the duration of the symptoms and any antibiotic therapy that has already taken place. Utilizing tests that have been available up to now, the demonstration of antibodies is successful in only 20-50% of the cases with erythema migrans, in 50-90% of cases with neurological manifestations, and in 90-100% of

the cases with ACA and arthritis.

Treatment of lyme-borreliosis is primarily carried out with penicillin G, tetracyclins, erythomycin or cephalosphorins. Lyme-borreliosis often heals spontaneously in the early stages, although later manifestations cannot be ruled out. For this reason, treatment in the earliest stages is indispensable. Moreover, a clinical cure following antibiotic treatment during the later manifestations is achieved in only a portion of the cases (for example, only about 50% for lyme-arthritis).

For this reason, lyme-borreliosis should be diagnosed at the earliest possible moment. Since (as already mentioned) the isolation of the agent is expensive, time consuming and not always successful, better serodiagnostic tests should be developed. The procedures used up till now (immunofluorescence test (IFT), indirect haemagglutination test (IHA), enzyme-linked immunosorbent assay (ELISA)) often fail in the early stages. The antigens for these tests are whole B. burgdorferi cells or ultrasonically treated whole cells. The use of different B. burgdorferi strains as antigens leads to varied test results in the ultrasonic-ELISA. The cells are fixed on an object slide (or the ultrasonic antigen is fixed to microtiter plates), and incubated with serum or liquor. The borrelia-specific antibodies are detected with a second fluorescence-marked or peroxidase-marked antibody of the corresponding immunoglobulin class. The reaction is then evaluated either in a fluorescence microscope (IFT), or according to a colour relation in a photometer (ELISA).

A problem for the specificity of the tests relates to the broad cross-reactions of the agent B. burgdorferi with other bacterial agents, particularly T. pallidum, the agent of syphilis. Since the test antigen consists generally of lysates of the entire agent, antibodies against the so-called "common antigens" are also picked up (Hansen, K.; Hindersson, P.; Pedersen, N.S. (1988): Measurement of antibodies to the Borrelia burgdorferi flagellum improves serodiagnosis in Lyme disease. J.Clin.Microbiol., 26, 338-346). "Common antigens" are widely distributed, and in their sequence, strongly preserved proteins, i.e. the "common antigens" of borrelia, treponema, and numerous other bacteria as well, have common epitopes. Moreover, false positive reactions can arise in IgM-IFT or IgM-ELISA from sera with rheumafactor activity. Therefore in order to make tests more specific, upon evidence of IgG- and IgM-antibodies a pre-absorption of the sera with an ultrasonically treated treponema, and in addition upon evidence of IgM antibodies a further absorption with rheumafactor absorbent, is carried out.

Therefore it is an object of the present invention to make available immunologically active proteins of Borrelia burgdorferi which can be used in a test kit that does not suffer from the disadvantages mentioned above. Furthermore, this test kit will produce quick and reliable evidence of antibodies directed against Borrelia burgdorferi.

A further object of the present invention is to make available monoclonal antibodies which are directed against specific

immunologically active proteins of Borrelia burgdorferi. Moreover, immunologically active proteins are made available which are suitable as vaccines against infections caused by borrelia strains.

During investigation of patient sera from different illness stages of lyme-borreliosis using the Western Blot, as well as the investigation of non-lyme-borreliosis patients (particularly syphilis patients) as to cross-reactivity with B. burgdorferi, immunologically active proteins (B. burgdorferi antigens) were found which on the one hand give rise to good antibody response following infection, and on the other hand show limited cross-reactivity with non-B. burgdorferi-positive sera (Example 1). It was shown that a particular strain of B. burgdorferi with the internal laboratory identification PKo, which was deposited with the German microorganism bank (DSM) under No. 5662, contains inter alia an immune-dominant protein in the molecular weight region of approximately 22kD, pC-protein). The determination of the molecular weight of the protein in accordance with the invention was accomplished using methods known per se, particularly using SDS-gelelectrophoresis. It was found that this protein was immuno-dominant for the IgM response. This protein is not expressed in the same manner in all B. burgdorferi strains. In accordance with the invention, this immunologically active protein (pC-protein) is gene-technologically created (Example 3).

Also, other immunologically active proteins (antigens), suitable in particular ways for use in test kits, were produced in generally and commercially available Escherichia coli cells, for

example the strains JM 105 (Pharmacia) or DH 5 (Gibco-BRL). B. burgdorferi DNA fragments coding for these proteins were isolated, and subsequently installed in effective expression vectors (Examples 2 and 3).

The identification and isolation of corresponding DNA fragments was carried out according to various methods. For example, an immunologically active protein with a molecular weight of approximately 41kD, hereinafter referred to as p41-protein, was produced with a polymerase chain reaction (PCR) and specific primers whose sequences were synthetically manufactured (Example 2).

Further there was located a gene bank for the B. burgdorferi genome, which was searched, utilizing monoclonal antibodies, for the direct expression of immunologically active proteins.

In a similar manner, proteins with molecular weights of about 100kD and 31kD were also cloned and sequenced.

A further method consisted of purifying particular selected immunologically active proteins (antigens) from B. burgdorferi lysates and then ascertaining the amino acid sequences of these antigens. Subsequently, the oligodesoxynucleotides corresponding to the amino acid sequence was synthesized, and identified by the hybridisation of those clones of the gene bank which showed DNA sequences coding for the immunologically active proteins. Both of the last-named methods are further described in Example 3.

After characterization, sequencing and cloning of the genes in corresponding expression vectors, the antigens in E. coli cells

were expressed and subsequently purified. A preferred cleaning process is described in Example 4.

The immunologically active proteins of Borrelia burgdorferi, in accordance with the invention, can be used in test kits which provide a surprisingly sensitive indication of antibodies against B. burgdorferi in different test liquids. The immunologically active proteins manufactured in accordance with the invention have the advantage that the preparations consist only of the desired protein and possibly those proteins which can be traced back to indications of degradation and/or incomplete translation. These preparations contain no B. burgdorferi proteins which do not correspond to the recombinant protein, since they were produced gene-technologically.

The term "test kits" is to be understood to refer to a set of test reagents which allow particular antibodies to be identified. The principles underlying the test kits were described in "Immunoassays for the 80s" (1981) by A. Voller et al., published by MTP Press, Falcon House, Lancaster, England. The test reagents contain, as the most important component, the antigen and possibly specific, preferably monoclonal antibodies.

The inventive test kits for identifying antibodies against Borrelia burgdorferi are characterized in that they make available at least one immunologically active protein which is without contamination by other proteins from the Borrelia burgdorferi strain. This immunologically active protein acts as an antigen and reacts with the antibodies available in the liquid under

examination. Preferably, the inventive test kits contain from two to four immunologically active proteins which are available without contamination by other proteins from B. burgdorferi. Furthermore, the test kits includes an indicator component which makes it possible to detect the presence of complexes of antigen and antibody.

The inventive test kits can be based upon various principles known per se. Essentially, the antigen can carry a marker which can be a radioactive isotope or an enzyme which catalyses a colour reaction. Likewise, the antigen can be bound to a fixed substrate (microtiter plates or spherule) and the indicator component can consist of an antibody-antagonistic antibody which carries a marker. The marker can consist of a radioactive isotope or an enzyme which catalyses a colour reaction.

Within the framework of the present invention, a preferred test kit is the so-called ELISA (enzyme-linked immunosorbent assay). An embodiment thereof is more fully described in Example 5. The results of this example show, surprisingly, that the use of only one immunologically active protein in accordance with the invention allows a very high specificity to be attained by the test kit. Moreover, the inventive test kits, surprisingly, make possible a differentiation correlating with the stage of the illness. The combined use of several antigens in a test kit makes it possible to identify antibodies against Borrelia burgdorferi in cases where the illness symptoms have not yet clinically manifested. Likewise, infection with B. burgdorferi can be

diagnosed when the patient shows only a subclinically infection. The evidence which can be obtained using the test kits in accordance with this invention is particularly significant in those cases where a tick bite can be established, but where it is not clear whether infection with a Borrelia strain has taken place.

Within the framework of the present invention, the combined use of several of the immunologically active proteins is preferred. Particularly preferred is a combination of proteins p41, pC, p17 and/or p100. By the use of the preferred ELISA test kits according to the invention, it is also possible to differentiate with respect to the nature of the antibodies. For example, if there is evidence of IgM antibodies, the so-called μ-capture-assay can be utilized. In this situation, the antibodies antagonistic to IgM antibodies are bound in the stable phase. After incubation of the test plates with the fluid under study, the IgM antibodies present in the test liquid are locked up in the stable phase. After saturation of non-specific bonds, an immunologically active protein according to the invention can be added. This antigen will then be indicated by a marker molecule. The antigen can be biotinylised, after which there is added Avidin which includes covalently bound peroxydase. The peroxydase catalyses a reaction leading to the development of colour.

A further possibility lies in that such monoclonal antibodies can be added to the complex: carrier-anti-IgM-antibody/antibody to be indicated/antigen in accordance with the invention, which are specific for the antigen and are biotinylised. Biotinylisation is

for example described in Monoklonale Antikorper, (1985) Springer Verlag, J.H. Peters et al. Evidence of the complex is achieved by adding avidin to which is coupled an enzyme which catalyses a colour reaction.

According to another embodiment of the present invention, the evidence of IgM is obtained through an indirect ELISA. The antigens in accordance with the invention are secured to microtiter plates and incubated with the liquid being studied. After washing one finds evidence of the immunocomplex by means of anti-μ-konjugate.

A further aspect of the present invention lies in the creation of monoclonal antibodies which are directed against the immunologically active proteins of Borrelia burgdorferi. The creation of such monoclonal antibodies is described in greater detail in Example 6. Such monoclonal antibodies can be used as reagents for direct evidence of the agent. It is also possible, however, to couple monoclonal antibodies in the stable phase to a microtiter plate. After addition of the immunologically active proteins (antigens), the latter are bound to the microtiter plate by antibody-antigen bonds. Next, the liquid to be investigated is added (which may for example be serum or liquor). The antibodies available in the test liquid then bind themselves to the antigen and can be detected with the help of a detection component.

Moreover, the monoclonal antibodies can be used quite effectively for purifying the immunologically active proteins (antigens). It is of advantage that the purification be

accomplished particularly carefully. The monoclonal antibodies are bound to a solid matrix. Preferably, this solid matrix is in the form of a column. Next the partially pre-perified antigens are mixed under physiological conditions with the antibodies coupled to a stable matrix. After washing the matrix-antibody-antigen-complex, the antigens can be elutriated. This would commonly be done using high salt concentrations or buffers with a pH value which facilitates the elutriation.

According to a further aspect of the present invention, DNA-sequences are made available which correspond entirely or partly with the amino acid sequence of the immunologically active proteins. These DNA sequences can be used preferably to obtain evidence of Borrelia strains in the investigation material through hybridisation. For this, an oligonucleotide is provided which partly corresponds to the DNA sequence. This oligonucleotide is radioactively marked. On the other hand, the DNA from the test material can be fixed to a suitable filter, preferably nitrocellulose, and subsequently hybridised with the radioactively marked oligonucleotide. Likewise, the DNA sequence in accordance with the invention can be utilized for in situ hybridisation in order to obtain direct evidence of B. burgdorferi in infected tissues. Instead of the chemically synthesised oligonucleotide, corresponding DNA fragments in bacteria can be multiplied and subsequently cut out of the vectors with the help of restriction endonucleases. After isolation of these DNA fragments, they can be radioactively marked and used for hybridisation as described above.

A further aspect of the present invention is that the immunologically active proteins (antigens) of Borrelia burgdorferi, in accordance with the invention, can be utilised as a vaccine. For this, the antigens in accordance with the invention are produced in a purified form. Next, they are used either alone or in combination, either with or without the agent which stimulates the immune response, to inoculate the patient. This process encourages the manufacture of antibodies which are specific against Borrelia burgdorferi strains.

The proteins, DNA sequences and monoclonal antibodies provided by the invention can be utilised in various areas. For example, the test kits according to the invention can also be used to obtain evidence of infection of animals by B. burgdorferi, and the proteins can also be utilised to inoculate animals, particularly valuable animals.

To the extent that the present invention relates to proteins of Borrelia burgdorferi, the test material can also be protein fragments which contain only a partial sequence of the complete amino acid sequence. Such partial sequences typically include at least ten amino acids and preferably at least fifteen amino acids.

Commonly, however, the protein fragments are larger. For example, it has been determined that, for a protein with a molecular weight of approximately 41kD, a deletion at the protein N-terminal of approximately 20 to 25 amino acids leads to a protein which shows an increased specificity. The reason for this could be that a so-called "common epitope" is deleted and specific epitopes

remain. The use of such deleted proteins is particularly preferred.

Within the framework of the present invention, proteins with a molecular weight of about 22kD to about 100kD are particularly preferred. These proteins can also be derived from other Borrelia burgdorferi strains.

Utilising the following tables, figures and examples, the preferred embodiments of the present invention will be fully described.

Example 1:

Identification of immune-relevant and Genus-specific Borrelia proteins.

Specific, commonly found serum antibodies were sought, which are directed against specific individual B. burgdorferi antigens, show the least possible cross-reactivity with proteins of related agents and also permit a correlation with the individual stages of lyme-borreliosis disease.

The search for commonly recognized antigens is carried out using Western Blot. A bacterial extract of B. burgdorferi (strain PKo) (Preac-Mursic, V.; Wilske, B.; Schierz, G. (1986): European Borreliae burgdorferi isolated from humans and ticks - culture conditions and antibiotic susceptibility, Zbl. Bakt. Hyg. A 163, 112-118) after pelletizing, resuspension in PBS/NaCl and ultrasonic treatment in SDS-polyacrylamide gel, was electrophoretically cleaved (Laemmli, U.K. (1970): Cleavage of structural proteins

during the assembly of the head of bacteriophage T4. Nature 227, 680-685).

The gels consisted of a collection gel with pockets for probes and a separation gel. The composition of the separation gels was the following: 15% acrylamide (Bio-Rad), 0.026% diallyltartardiamide (DATD, Bio-Rad) per percent of Acrylamide, 0.15% SDS, 375mM Tris-HcL pH 8.5, 0.14mM ammonium peroxodisulphate (AMPER, Bio-Rad) and 0.035% N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad). AMPER and TEMED served as radical starters for the polymerisation. From 2 to 4 hours after polymerisation, the collection gel (3.1% Acrylamide, 0.08% Diallyltartardiamide, 0.1% SDS, 125mM Tris-Hcl pH 7.0, 3mM AMPER and 0.05% TEMED) was poured over the separation gel and provided with a teflon ridge. The anode and cathode chambers were filled with identical buffer solution: 25mM Tris-Base, 192mM Glycine and 0.1% SDS, pH 8.5.

A 20 μl sample in lysis buffer (3% saccharose, 2% SDS , 5% β-mercaptoethanol, 20mM Tris-HCl, pH 7.0, bromphenolbleue; heated for 5 minutes at 100°C.) was applied to each pocket. The electrophoresis was carried out at room temperature overnight with a constant current strength of 6mA for gels having a size 20x15cm. Subsequently, the gels were transferred to nitrocellulose (NC).

For the protein transfer, the gel with adhering nitrocellulose was located between Whatman 3MM filter paper, electrically conductive foam 1cm thick, and two carbon plates which conducted the current along platinum electrodes. The filter paper, foam and nitrocellulose were well saturated with Blot-buffer (192mM glycine,

25mM Tris-Base, 20% methanol, pH 8.5). The transfer took place at 2 mA/cm^2 for two hours. Free linkage locations in the nitrocellulose were saturated for one hour at 37°C. with Cohen-buffer (1 mg/ml ficoll 400, 1 mg/ml polyvinylpyrolidone, 16 mg/ml of cow serum albumin, 0.1% NP 40, 0.05% bacto-gelatine in sodium borate buffer pH 8.2); (Cohen, G.H., Dietzschold, B., Ponce de Leon, M., Long, D., Golub, E., Varrichio, A., Pereira, L. and Eisenberg, R.J.: Localisation and synthesis of an antigenic determinant of Herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibodies. J. Virol. 49 (1984) 4183-4187). The Blot was incubated with vibration overnight at room temperature with the patient sera (dilution 1:100 in 154mM NaCl and 10mM Tris-NCl pH 7.5).

Following the serum incubation, the Blot was washed four times for 15 minutes each with TTBS (50mM Tris-NCl pH 7.5, 500mM NaCl, 0.01% Tween 20). Next, the Blot was incubated for two hours at room temperature with peroxidase-coupled anti-human immunoglobulin (DAKO, Hamburg, dilution 1:1000 in 154mM NaCl and 10mM Tris-HCl, pH 7.5) After several washes with TTBS the Blot was coloured with 0.5 mg/ml diaminobenzidine and 0.01% hydrogen peroxide in 50mM Tris-NCl pH 7.5. Next the colouring was halted with 1N sulphuric acid, whereupon the Blot was washed with water until free of acid and then dried between filter papers.

A selection of the reaction patterns of various sera with the Western Blot stripes is shown in Figures 1, 2a and 2b.

The following proteins showed themselves to be immune-

dominant: p17 (17kDa), pC (22kDa) p41 (41kDa) and p100 (100kDa with size variation in various B. burgdorferi isolates). Up to p41, the biological functions of these antigens are unknown; p41 produces the flagellin protein (Barbour, A.G.S., Hayes, S.F., Heiland, R.A., Schrumpf, M.E. and Tessier, S.L.: A Borrelia genus-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. 52 (1986) 549-554).

On the basis of these analyses, which were carried out with a larger number of patient sera from various stages of the illness, it appeared that not all of those infected with B. burgdorferi were subject to a given antigen. It appeared that, particularly for sera with IgM antibodies (fresh infection) next to the flagellin (p41) a further protein (pC) in the 22kD region was recognized quite frequently. The simultaneous appearance of both antibodies however was not inevitable. Sera were found containing only antibodies against p41 or only antibodies against the pC protein (Figures 1, 2a, Western Blots). In neuroborreliosis the evidence of the intrathecally formed antibodies in liquor is of great importance. IgG-Western Blots for 12 liquor-/serum pairs of patients with a Bannwarth lymphocyte meningeoradiculitis showed in all cases a local, intrathecal immune response against p41. In the late stage, next to the IgG antibodies were found against flagellin, antibodies against proteins in the 100kD range (p100) and in the 17kD range (p17), whereas these were not or only seldom detectable in the early stages. Thus, antibody reactivity against the p17 proteins and the p100 proteins are good markers for the

attainment of stage III (Figure 2b, Western Blot).

With the help of these four antigens, an improved standardization of the tests can be attained.

The proteins p41, pC and p17 show only a small cross-reactivity to other bacterial strains, and the protein p100 has shown itself to be a genus-specific protein for B. burgdorferi specific epitopes. Table 2 (the reactivity of immune sera against various bacterial agents with proteins of B. burgdorferi) summarizes the cross-reactivity of sera against various related agents with B. burgdorferi antigens according to the Western Blot analysis.

During attempts to purify the four proteins (p41, pC, p17, p100) from B. burgdorferi extracts, it became evident that large amounts of initial material were necessary. Particular difficulties attended the cleaning of p100, which is under-represented in the overall extract. Since the cultivation is expensive, gene-technological possibilities should be investigated for the production of these antigens.

The analysis of patient sera using the Western Blot has shown that with a combination of gene-technologically produced p41, pC, p17 and p100 as antigen, virtually all positive sera can be identified, and furthermore a correlation with the stage of the illness can be given.

Example 2:

Gene-technological Production of p41 (flagellin) from B.

burgdorferi in Escherichia coli.

The coding region for p41 was determined using DNA-amplification employing a "polymerase chain reaction" (PCR) using a B. burgdorferi (DSM-No. 5662 P/Ko2/85) whole-DNA preparation. The obtained sequence was subsequently put under the control of induceable promotors and caused to be expressed after transfection into E.coli (Maniatis, T.; Fritsch, E.F.; Sambrook, J. (1982) Molecular cloning. Cold Spring Harbor).

For this, the B. burgdorferi cells were cultivated for two weeks at 37°C. in 2 l of modified barbour-stoenner-kelly-(BSK) medium (Preac-Mursic, V.; Wilske, B.; Schierz, G. (1986): European Borreliae burgdorferi isolated from humans and ticks - Culture conditions and antibiotic susceptibility. Zbl. Bakt. Hyg. A 163, 112-118), pelletized at 6000rpm, washed in TEN-buffer (10mM Trish-HCl pH 7.6; 1mM EDTA; 10mM NaCl), and resuspended in 20ml of Lysozym buffer (20% saccharose, 50mM Tris-HCl pH 7.6, 50mM EDTA, 5 mg/ml Lysozym). After incubation for 30 minutes at 37°C., protoplasm appearing on the cell wall due to the effect of the Lysozym was lysated by adding 1ml of 25% SDS (sodium dodecyl sulfate). After a further 10 minutes, 4ml of a 5 M NaCl solution were added. Protein was denaturized by the addition of an equal volume of TE-saturated phenol (TE: 10mM Tris/HCl, pH 7.8, 1mM EDTA). The separation of the phases was accomplished with centrifugation at 4°C. and 6500rpm for 5 minutes. The upper aqueous phase containing DNA was transferred carefully into a fresh capillary tube with a pipette having a wide opening (to avoid shear

forces), and thereafter again extracted with the same volume of phenol/chloroform/isoamylalkohol (1:1:0.04). After the separation, the upper aqueous phase was again carefully transferred to a new tube, and the DNA was precipitated with a double volume of ethanol. After about 5 minutes the DNA, precipitating as long, thread-like shapes, was removed by wrapping on a glass rod, and transferred into a 70% ethanol solution for washing. The DNA adhering to the glass rod was then stored for 2 hours at room temperature, in order to allow the ethanol to evaporate, after which the DNA was transferred into 4ml of TEN-buffer.

Every 1 µl of the B. burgdorferi whole DNA thus obtained was amplified in five 100µl PCR preparations.

As a specific primer for the polymerase-catalysed amplification, sequences were selected which contained the information for the translational start as well as the 3'-end of p41 (flagellin). The DNA-sequences used for this are shown in Figure 3. The two oligodesoxynucleotides were synthesized on a Milligen/biosearch DNA-synthesizer 8700 in 1 µmol columns, then after cleavage purified in a preliminary manner with ammonia by way of ethanol precipitation, and then each was dissolved in 400µl of H_2O. Each 1µl of this oligodesoxynucleotide solution was applied to a PCR preparation; the buffer, nucleotide and the Taq-polymerase came from a commercially available test kit (Cetus/Perkin-Elmer, Uberlingen) and were used in accordance with the test description. The temperature conditions for the individual cycles were:

2 minutes of denaturing at 94°C

2 minutes of annealing at 45°C

4 minutes of DNA-synthesis at 73°C

50 cycles were carried out.

The PCR preparations were then purified and the DNA was removed after the addition of NaCl in an end concentration of 0.2M with 2.5 volumes of ethanol at -20°C for 5 hours. After pelletizing and washing in 70% ethanol, the DNA was dissolved in 200 µl of H_2O, and then split after the addition of the corresponding buffer with the restriction enzymes Bam HI and Pst I (Boehringer Mannheim) in accordance with the instructions of the manufacturer.

After the gel-electrophoretic splitting in a 1.5% agarose gel, the amplified DNA fragment (about 1000bp) was isolated and inserted into a pUC8 vector (pharmacia) sectioned with BamHI and PstI (Vieira, J.; Messing, J. (1982): The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268), so that 0.25 µg of the vector, 0.5 µg of the p41 fragment and 2U of T4-DNA-ligase with buffer were used in accordance with the instructions of the manufacturer (Boehringer Mannhein).

After transformation of the ligated DNA fragments into the E. coli strain JM 109 (pharmacia) (Yanisch-Perron, C.; Vieira, J.; Messing, J. (1985): Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119), and streaking on agar plates with ampicillin (50 µg/ml) and X-Gal (30 µg/ml), white colonies were withdrawn in 5ml

of L-broth medium, and the isolated plasmids were investigated using restriction enzyme splitting at their inserts.

The B. burgdorferi flagellin coding DNA-fragment is located behind the inducible lacUV5 promotor of the vector in the same reading branch as the lacZα-coding transcript started by this promotor. By this means, a flagellin emerged, with contained at its N-terminus several pUC8-coding amino acids. This region is arranged as follows:

ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC ATC ATG ATT

MET THR MET ILE THR ASN SER ARG GLY SER ILE MET ILE

pUC8 p41

Liquid cultures were again set up with positive E.coli clones (for example, pUC8 ly13) which contained the vector with a DNA insert in the expected length (1000bp), and the transcription of the lac-promotor of the plasmid, was induced using a 3-hour induction with 1mM IPTG at 37°C and with vibration. 1.5ml of this culture were then briefly pelletized, the bacteria were lysated with "boiling mix" (3% saccharose, 2% SDS, 5% β-mercaptoethanol, 20mM Tris-HCl pH 7.0, 2% bromphenolblue) at 100°C for 10 minutes, and the proteins were separated using 17.5% SDS-PAGE. After colouring of the proteins with Coomassie brillant blue, there appeared in the cells with the plasmid insert a new, additional band at about 41kD, corresponding to the expected size of the flagellin. A specific reaction of this recombinant antigen with a serum from a lyme borreliosis patient as well as a monoclonal antibody against B. burgdorferi p41 flagellin, is indicated in the

immunoblot shown in Figure 4.

Any other induceable plasmid which starts a transcript in the same reading row is just as suitable as pUC8 for the production of p41. The expression of an authentic p41, not fused with any foreign amino acids, is also possible by splitting the p41-coding region at the translation start with BspHI (TC ATG A) and PstI (at the 3'-end) and installation of the fragment at the NcoI-position (CC ATG G) and PstI-position of a so-called ATG-vector.

For the process referred to below, the clone pUC8ly17 was used.

Example 3:

The Production of pC,OspA and p100 in E.coli from B. burgdorferi gene banks.

For the preparation of B. burgdorferi-specific DNA-sequences, a chromosomal gene bank was applied to E.coli. With the help of suitable methods, such as immuno-screening or hybridization with selected oligonucleotides, it was possible to identify, in this gene bank, E.coli clones which contained corresponding B. burgdorferi-specific DNA-sequences. Following restriction-enzyme analysis, a restriction-enzyme map was established. This could be used either to transfer the sought for DNA-sequences into expression vectors, or to establish the sequencing thereof. The detailed process: In order to isolate B. burgdorferi (DSM-No. 5662) DNA (chromosomal DNA such as plasmid DNA), the cells were cultivated as described in Example 2. After centrifugation at

12000Upm for 20 minutes, the cells were washed and resuspended in SET buffer (20% saccharose, 50mM Tris-HCl pH 7.6; 50 mM EDTA). The cell wall was partially split by adding 15mg/ 5ml of lysozym for 20 minutes. Next, the protoplastized cells were lysated by the addition of SDS (n-dodecylsulfate sodium salt) with a final concentration of 1%. After 20 minutes at 37°C, proteinase K (final concentration 1 mg/ml) was added twice for one hour each, and the DNA-containing solution was placed in 100mM NaCl with TEN-buffer (10mM Tris-HCl pH 7.6, 1 mM EDTA, 300mM NaCl). This was followed by a phenol extraction and two further phenol/chloroform/iso-amylalcohol-extractions (phenol:chloroform in the ratio of 1:1; chloroform:iso-amylalcohol in the ratio of 24:1). The extracted remnant was removed with 2.5 volumes of 95% ethanol, and the DNA was precipitated at -20°C. The thread-like precipitated DNA was recovered by winding on a glass rod, and washed in 70% ethanol. After a brief drying in the exsiccator, the DNA was placed in TE-buffer (10mM Tris-HCl pH 7.6, 1mM EDTA) which contained RNAse (20 ug/ml). The DNA thus prepared was used for the subsequent steps.

B. burgdorferi DNA was incubated with the restriction enzyme Sau 3A (Boehringer Mannheim) according to the instructions of the manufacturer. By selecting appropriate enzyme dilutions and reaction time of the enzyme on DNA, a partial splitting of the latter was attained. The so-obtained, partially split DNA was ligated with vector DNA (pUC18 or other suitable DNA vector) which was BamH I-restricted and dephosphorylized by treatment with alkali phosphatase. Next T4 DNA-ligase (Boehringer Mannheim) was added

according to the instructions of the manufacturer. 0.2-0.5 µg/µl of whole DNA was applied per transformation preparation. E.coli JM 109 (or other suitable E.coli strains) were transformed with the ligated DNA according with the protocol of Hanahan (Hanahan, D. (1985): Techniques of Transformation of Escherichia coli, S. 109-115. In: D.M. Glover (Hrsg.) DNA cloning, Vol. 1. A practical approach. IRL Press, Oxford bzw. nach Maniatis et al. (Maniatis, T. (1982): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Recombinant E.coli clones were selected and cultivated on LB-medium (10g Trypton, Difco, 5g yeast extract, Difco, 5g NaCl, Merck), containing 100 µg/ml ampicillin (or another suitable antibiotic). The colony pattern was identically transferred to LB-plates followed by colony expression on nitrocellulose. The cells from these colonies were differentially lysed on a filter, according to the screening process utilized. With the use of mono- or polyclonal sera (immunoscreening) for the detection of B. burgdorferi specific gene products induced by the recombinant DNA, the cells were lysed for 15 minutes over saturated chloroform vapor. After saturation of the thus-treated filter with a skim milk solution for 2 hours, the filters were incubated over night with the various sera, washed repeatedly with TTBS-buffer (s.o.) and incubated for 2 hours with the second peroxidase conjugated antibody (Dako, Hamburg). Additional washing with TTBS-buffer served to reduce the non-specific, bound, peroxidase-conjugated antibodies. By the enzymatic transformation of the substrate diaminobenzidine (Sigma-

Chemie, Munchen) and H_2O_2 into an insoluble brown pigment, it was possible to recognize positive E.coli clones, i.e. B. burgdorferi antigen-producing E.coli clones. The thus recognized positive E.coli clones were inoculated from the initial plates and analysed. By the use of specific oligonucleotides for hybridization and thus the detection of specific B. burgdorferi antigens sequences (screening by hybridization), the cells were lysed in an alkali environment (by wetting the filter for 5 minutes with 0.5 M NaOH, 1.5 M NaCl) on the nitrocellulose filter (Schleicher & Schuell). After neutralization (through moistening the filter for 5 minutes in 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) the filters with the denatured DNA was wetted with 2x SSPE (20x SSPE: 3.6 M NaCl, 200 mM NaH_2PO_4, pH 7.4, 20mM EDTA, pH 7.4) and then dried. The DNA was then fixed by baking the filter for 2 hours at 80°C. The filters thus treated were then used for the hybridization. The hybridization took place with the use of radioactive (^{32}P) or non-radioactive (for example, digoxigenin, Boehringer Mannheim) detection methods. The process used either known marking methods (Maniatis, T. (1982): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor) or marking methods recommended by the manufacturer (Boehringer Mannhein) (^{32}P-marking with ^{32}P-gamma-ATP and kinase-reaction or digoxygen-marking with Dig-11-UTP and terminal transferase reaction). A restriction-enzyme analysis was carried out on positive E.coli clones, and with this information an expression of the antigen-coding DNA sequences in suitable vectors, or their sequencing, was carried out.

In the hybridization tests, synthetic oligodesoxynucleotides were initially used, whose sequence on the basis of (...) were selected from p100 and pC amino acid-sequences.

The following process took place:

From lysates of B. burgdorferi, both proteins were partially purified by extraction with n-Octyl β-D-thioglucopyranoside and then cleaved using SDS-polyacrylamide-gel-elektrophoresis. Next the antigens were transferred to a glass fibre matrix using Western Blot, and the corresponding portions with the B. burgdorferi-proteins were cut out. p100 was then N-terminal sequenced, and the first 22 amino acids of the amino terminal were determined (these methods of "micro-sequencing" are described in: Eckerskorn, C., Mewes, W., Goretzki, H. and Lottspeich, F.: A new siliconized fibre as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. 176 (1988) 509-519). Using pC, a direct sequencing was not possible, since the N-terminal of a sequence is not directly accessible, i.e. a Myristillation or similar modifications may be required for this. Therefore, this protein was tryptically split, the fragments cut away using HPLC, and two of them were then sequenced. The subsequent oligodesoxynucleotide sequences were then derived from the thus determined amino acid sequences. Since several codon-possibilities exist for an amino acid, it is necessary to keep in mind the base variations at the corresponding position of the oligonucleotide, and during the synthesis to respect equimolar ratios.

p100-p1 - p100 - Amino Acid Sequence:

Glu Leu Asp Lys Glu Lys Leu Lys Asp Phe Val Asn Leu Asp Leu Glu Phe Val Asn Thr

p100 - oligodesoxynucleotide sequence, given in parentheses, and bases separated by ";", were utilized during the synthesis (in a Milligen/biosearch 8700 DNA Synthesizer) in equimolar ratios:

GA(G;A) (C;T)T(G;T;A) GA(C;T) AA(G;A) GA(G;A) AA(G;A) (C;T)T(G;T;A) AA(G;A) GA(C;T) TT(C;T) GT(T;A) AA(C;T) (C;T)T(G;T;A) GA(C;T) (C;T)A(G;T;A) GA(G;A) TT(C;T) GT(T;A) AA(C;T) TA(C;T) A

The oligodesoxynucleotide sequence was used as a probe, and hybrdized with clones containins the B. burgdorferi DNA. After sub-cloning, a clone was obtained which contained the gene for p100. The following coding DNA sequence of p100 (5'-end) of the strain PKo was determined having a length of 346 base pairs:

5' ATG AAA AAA ATG TTA CTA ATC TTT AGT TTT TTT CTT GTT TTT TTA AAT GGA TTT CCT CTT AAT GCA AGG GAA GTT GAT AAG GAA AAA TTA AAG GAC TTT GTT AAT ATG GAT CTT GAA TTT GTT AAT TAC AAG GGT CCT TAT GAT TCT ACA AAT ACA TAT GAA CAA ATA GTA GGT ATT GGG GAG TTT TTA GCA AGG CCG TTG ATC AAT TCC AAT AGT AAG TCA AGT TAT TAT GGT AAA TAT TTT GTT AAT AGA TTT ATT GAC GAT CAA GAT AAA AAA GCA AGT GTT GAT ATT TTT TCT ATT GGT AGT AAG TCA GAG CTT GAT AGT ATA TTA AAT CTA AGA AGA ATT C... 3'

After complete cloning, the following amino acid sequence was determined:

(insert 25a)

Amino Acid sequence of p100 protein

In a similar manner by way of the pC-amino acid sequences:

p1: Lys Ile Thr Asp Ser Asn Ala Thr Val Leu Ala Val Lys

p2: Asp Leu Phe Glu Ser Val Glu Gly Leu Leu Lys

the corresponding oligodesoxynucleotide sequences were synthetized:

pC-p1-oligodesoxynucleotide-sequence:

AA(G;A) AT(T;A) AC(A;T) GA(T;C) (A;T)C(A;T) AA(T;C) GC(A;T)
AC(A;T) GT(A;T) (T;C)T(G;A;T) GC(A;T) GT(A;T) AA(A;G) A

pC-p2-oligodesoxynucleotide-sequence:

GA(T;C) (C;T)T(G;A;T) TT(T;C) GA(G;A) (T;A)C(A;T) GT(A;T)
GA(G;A) GG(A;T;C) (T;C)T(G;A;T) (T;C)T(G;A;T) AA(A;G) A

Following the discovery of suitable clones by way of hybridization and sub-cloning of the desired gene, the following coding DNA-sequence of pC of the strain PKo, with a length of 639 base pairs, was determined:

(insert bottom of p.26)

The protein pC with a length of 212 amino acids has the following sequence:

(insert from p.2 at top)

Amino acid sequence of pC protein - 22kD -

In a corresponding manner, a portion of the coding DNA sequence of OspA (5'-end) of the strain PKo, with a length of 680 base pairs, gave the following:

(insert from p. 27 at bottom of page)

After complete sequencing, the following amino acid sequence for the 31 kD protein was established:

(insert from p.28 at top)

Amino acid sequence of OspA (strain Pko)

Example 4:

Purification of the Recombinantly Produced B. burgdorferi Antigens

a) Example of p41 (flagellin)

A 50ml overnight culture of the clone pUC8ly2 described in Example 2 was placed in 1.5ml of fresh L-broth medium and incubated at 37°C under intensive vibration. Upon reaching an optical density of 0.7, the culture was further incubated for 3 hours with IPTG in an end concentration of 1mM. The bacteria were pelletized (6000rpm, 10 minutes), resuspended in 300ml 20mM Tris-HCl pH 8.0, 50mM EDTA, 0.5 mg/ml lysozyme, and placed in a 37 degree C water bath for 45 minutes. Following addition of NaCl in an end

concentration of 150mM and triton-X-100 in an end concentration of 1%, further incubation took place for 45 minutes at 37°C, where upon the suspension was treated ultrasonically three times for five minutes each. Insoluble components were pelletized at 9000rpm for 30 minutes, resuspended in 20mM Tris-HCl pH 8.0, 10mM dithiothreitol and 1% octyl-gluco-phyranoside (Sigma-Chemie, Munchen), and stirred for one hour at room temperature. After subsequent pelletizing of the insoluble components at 1700rpm for 30 minutes, the supernatant was carefully decanted.

Subsequently, the pellet was resuspended in 150ml 20mM Tris-HCl pH 8.0, 8 M urea, 1% β-mercaptoethanol by stirring for 2 hours. Here as well, insoluble components were removed through centrifugation at 1700rpm for 30 minutes, and the supernatant liquid was pumped into a DEAE-sephacel-column (Pharmacia, Freiburg) with a gel volume of 550ml (3cm diameter, 80cm high). The elution of the p41 antigen took place in a NaCl-gradient of 0-800mM in a total volume of 600ml. The recombinant p41 was elutriated at an NaCl-concentration of approximately 0.25 M. The corresponding fractions were combined, and further cleaned using an HPLC with a mono Q column (anion exchanger) (Figure 4). An elution profile with the cleaned p41 in an NaCl-gradient from 0-800mM is shown in Figure 5. The p41-positive fractions (according to Western Blot analysis) were dialysed with 20mM Tris-HCl pH 8.0, 10mM $MgCl_2$ and 0.1% β-mercaptoethanol, and then used for the tests indicated in Example 5. By purification of the p41 derived from 1 litre of bacterial culture, a yield of 5 to 10mg can typically be expected.

After sequencing, the following amino acid sequence was established:

(insert on 29a)

Amino acid sequence of p41 protein

b) Purification of Recombinant Borrelia burgdorferi Antigen pC from E.coli

A clone containing the gene for the antigen pC (pDS1PC5) was inoculated into 100ml of L-broth (with 50 ug Ampicillin/ml), left overnight to grow, and then transferred into 900ml of L-broth/ampicillin - double concentrated yeast extract/2ml glycerin, induced after about 1 hour with 2mM IPTG, and agitated for 2-3 hours further.

After centrifugation at 8000rpm for 10 minutes, the pellet was resuspended in 20ml Lyse-buffer (50mM Tris-HCl, pH 7.5, 2mM EDTA, 0.1mM DTE, 0.1mM PMSF; 0.4 mg/ml lysozyme). After 30 minutes of stirring at room temperature, Triton-X-100 (end concentration 0.1-0.2%) was added. 10 ul of benzonase (Merck) was also added. This was followed by a further 30 minutes of stirring at room temperature. The now clear suspension was fixed with solid NaCl at 1 M NaCl, and stirred for a further 30-60 minutes at 4°C.

After centrifugation at 4°C for 30 minutes and 15,000rpm, a quantity of the pC-protein had entered the supernatant. The pellet was discarded. The supernatant liquid was dialysized using 10mM Tris-HCl, pH 8.0, with repeated buffer exchange. After centrifugation and/or filtration (it) was applied to DEAE-sepharose (Pharmacia), so that the column was equilibrated with 10mM Tris-HCl, pH 8.0. Upon elution with 0 M NaCl, the pC-protein was found at the second peak of the run. The first fraction was discarded, while the rest was collected and re-chromatographed. The

separation column was regenerated with 1 M NaCl, and equilibrated in 10mM Tris-HCl pH 8.0. The antigen thus developed can now be used in a suitable test kit, for example ELISA.

c) Purification of Recombinant Borrelia burgdorferi Antigen OspA from E.coli

A clone containing the gene for the antigen OspA (pDS10spA) was inoculated into 100ml of L-broth (with 50 µg ampicillin/ml) and left overnight. The culture liquid was transferred into 900ml of L-broth/ampicillin - double concentrated yeast extract/2ml glycerin, induced after 1 hour with 2mM IPTG, and for 2-3 hours further agitated.

The cells are centrifugated at 6000rpm for 5 minutes and the pellet resuspended in 20ml Lyse-buffer (50mM Tris-HCl, pH 7.5, 2mM EDTA, 0.1mM DTE, 0.1mM PMSF; 0.4mg/ml lysozyme). After 30 minutes of stirring at room temperature, Triton-X 100 (end concentration 0.5-1%) was added. Also added were 10µl benzonase (Merck). Then further stirring took place for 30 minutes at room temperature.

The now clear suspension was fixed with solid NaCl at 1 M NaCl, and further stirred (at 4°C). After centrifugation at 4°C for 3 minutes and 15,000rpm, the OspA was essentially in pellet form. The supernatant liquid was discarded and the pellet was resuspended in 2 M urea (with 50mM Tris-HCl, pH 7.5, 2mM EDTA, 0.1mM DTE). The OspA is now in the supernatant liquid.

The supernatant liquid is dialysated in a dialysis tube with

5mM MES (2-[N-morpholino]ethane-sulfonic acid) buffer, ph 6.0, during which a repeated buffer exchange is unconditionally necessary. After centrifugation and filtration, the protein was placed in a S-sepharose fast flow (Pharmacia) column. First, washing took place with 0 M NaCl and then elutriation with a gradient of 0 to 1 M NaCl. The OspA-antigen elutriated as a sharp peak at approximately 0.4 M NaCl. After dialysis with 10mM-Tris-HCl pH 7.5, the OspA antigen can be used in a suitable test kit, for example an ELISA.

Example 5:

The Use of Recombinantly Produced B. burgdorferi Antigens (Example p41) in ELISA.

Conditional upon the high purity of the produced recombinant antigens, specific tests are possible for B. burgdorferi which are machine-readable and which can be carried out without great expense in the technical and personnel areas.

Microtiter plates were coated each with 50 µl of purified p41 (concentration 0.5-5 µg/ml). In accordance with standard methods, the plates were incubated overnight at 4°C and washed, whereupon the bond locations still free were saturated with a 2% cow serum albumin solution. Next, each plate received, by pipette, 50 ul serum (dilution 1:200), then incubated for 2 hours at 37°C. The unbound portions were washed away and the bound immune complex was detected with 50 µl of peroxidase-marked anti-human IgG (dilution

1:1000) per plate. After a further washing, the plates were each coated with the 100 µl ortho-phenylene diamine (concentration 0.1% in 0.1 M phosphate buffer pH 6.0 with 0.03% H^2O^2) as a colouring reagent, whereupon the colouring process took place in the dark for 10 minutes upon which it was arrested with 100 µl 1 N sulphuric acid. The microtiter plates were evaluated in a photometer at 486nm (Figure 6).

In the example here shown, 7 positive and 8 negative anti-B. burgdorferi sera were tested. Of the clinically guaranteed lyme-positive sera, there were three which showed no reaction with p41 using Western Blot stripping with B. burgdorferi as antigen, thus pointing out the sera from the early stage of the infection. These reacted in ELISA only marginally with the recombinant antigen. Normal p41-positive sera, by contrast, reacted quite well, whereas lyme-negative sera remained in the region under an OD of 0.3.

Example 6:

Manufacturer of B. burgdorferi Specific Monoclonal Antibodies

Female Balb/c mice were intraperitoneally immunized with B. burgdorferi (DSM-No. 5662). The first immunization took place with complete Freund Adjuvans, with 2 to 5 immunizations with incomplete Freund Adjuvans at intervals of two weeks. Two weeks later, the antigen was applied without Adjuvans, and three days later the mice were killed and the spleen removed.

The spleen-lymphocytes were mixed with mouse-myeloma cells

(Ag8-653) at the ratio of 1:1, settled out, and mixed with a fusion solution (2.5g polyethylene glycol (PEG), 2.5ml RPMI-medium, 250 µl DMSO): one minute for the addition of the fusion solution, 90 seconds of incubation at 37°C. The cells were then again settled out, where upon the PEG was removed and added to a culture medium (HAT-medium). Finally, the cell suspension was distributed to microtiter plates which contained macrophages as feeder cells, and then incubated. The hybridoma supernatant was tested undiluted by indirect immunofluorecence (IFT) (Wilske, B.; Schierz, G.; Preac-Mursic, V.; Wever, K; Pfister, H.-W.; Einhaupl, K. (1984): Serological diagnosis of erythema migrans disease and related disorders. Infection,12, 331-337).

IFT-positive cell supernatant was analyzed using the Western Blot. Hybridomas reactive in the Western Blot were four times subcloned by way of "limiting dilution", and identified with respect to immunoglobulin-class and the IgG subclass.

In this manner, the following monoclonal antibodies (MAB) were obtained:

1. MAB against p41:

   (a) L41 1C11

   This antibody was reactive with all 30 tested B. burgdorferi strains and with relapse fever Borrelia (except for B. hermsii), however not with treponemas.

   (b) L41 1D3

   This antibody was reactive with the majority (21 of 24) of the B. burgdorferi strains, but not with relapse fever

Barrelia or Treponemas.

2. MAB against p100 (L100 ID4):

This antibody was reactive with all 30 tested B. burgdorferi strains, however not with relapse fever Borrelia or treponemas.

3. MAB against pC (L22 1F8):

This MAB was reactive with pC-proteins from skin and liquor, although the pC-proteins were negative for some but not all tick strains.

4. MAB against OspA:

OspA is a primary protein (30kD-region) of the outer membrane of most B. burgdorferi strains. The OspA proteins of European B. burgdorferi strains are antigenetically heterogeneous and differ antigenetically from the American strains. The few OspA-negative strains have pC-proteins

(a) L 32 2E7

Altogether, 29 out of 32 strains were reactive. The negative strains show no OspA-protein. The three negative strains were reactive with the pC-specific MAB L22 1F8.

(b) L32 1G3:

This MAB was reactive with only 3 out of 25 tested strains.

The combination of MAB L32 2E7 and MAB L22 1F8, as well as the reaction with MAB L100 1D4, allowed the identification of B.

burgdorferi borrelias and treponemas. An absolute identification and differentiation of B. burgdorferi has not been possible with the monoclonal antibodies thus far available.

Example 7:

Determination of the Amino Acid Sequence of a Protein with a Molecular Weight of Approximately 22kD from Another Strain

In accordance with the methods described in the above examples, the amino acid sequence of a protein with a molecular weight of approximately 22kD was determined. This protein was cloned from another borrelia-strain, and subsequently sequenced. This strain was deposited under No. 35210 with the ATCC, and is generally available. The following amino acid sequence was determined.

(insert from p.36)

Amino acid sequence of PC protein

Example 8:

Comparison of Test Kits with Proteins in Accordance with the Invention, the Test Kits using Ultrasonics.

Tests were carried out on 74 sera of patients with erythema migrans, as to IgM-antibodies and IgG-antibodies. In addition a negative control group of 100 blood donors was tested. In these tests, ultrasonic preparations of Borrelia burgdorferi were treated once in accordance with the known ELISA-testing method. On the other hand, recombinant proteins prepared in accordance with the invention were separated and applied together. The following tables show clearly that the process in accordance with the invention attains a substantially higher sensitivity, by comparison with the use of ultrasonics.

RESULTS for IgM-Antibodies

(insert from p.37 at top)

RESULTS for IgG-Antibodies

(insert from p.37 at bottom)

RESULTS on IgG and/or IgM Antibodies

(insert from p.38)

Description of the Tables

Table 1:

Reactivity of lyme borreliosis-sera from various illness stages with B. burgdorferi-antigens (p17, pC, p41, p100) in the Western Blot with B. burgdorferi-lysate as antigen.

Table 1 provides an overview of the immune-dominant proteins in various stages of lyme borreliosis.

1.1 Sera from healthy persons, and to a greater degree sera of syphilis patients, show antibodies against p60 ("common antigen"). Only seldom with antibodies against p41 detected.

1.2 The immune-dominant proteins during the early stages (EM and LMR) were the flagella-protein p41 and the pC-protein. pC is the immune-dominant protein for the early immune response. In particular, IgM-antibodies against pC can appear earlier than IgM-antibodies against p41 (see also Figure 2a).

1.3 The sera of patients with late manifestations (ACA and arthritis) reacted in all cases (N=22) with p41 or p100, and in 21 cases with p100 or p17. p17 was reactive in 17 cases, p100 in 19 cases and p41 in 20 cases.

1.4 The intrathecal IgG immune response was directed against p41

in all 12 of the studied cases. Antibodies against p41 in serum were not detectable in three cases.

Table 2

The reactivity of immune sera (against various bacterial agents) with proteins of B. burgdorferi (Western Blot).

Western Blot stripes with electrophoretically cleaved B. burgdorferi-lysate were produced as described in Example 1, and incubated with sera against various, more or less related, and thus cross-reacting agents. The sera came from rabbits which had been immunized with the current agents. The lowest cross-reactivity was found with p100; only one (anti-B. hermsii) of the tested 15 agent-specific sera reacted with this protein. p41 and pC each react with three of the sera and therefore appear more suitable for diagnostic use. The presence of immune-conserved antigens was clearly shown; for example, 14 of the tested sera react with proteins of size 40kD, while 12 of the tested sera react with proteins of size 60kD (p40, p60). These "common antigens" are thus unsuitable for diagnostic use.

Table 1: Immune-dominant proteins for the (...) immune response (...) lyme borreliosis (original illegible - see top of p. 41)

1.1 Reactivity of Human Test Sera (IgG Western Blot)

	pC	p41	p60	Number
Healthy	.	2	3	17
Syphilis	.	1	5	9

1.2 Immune response against pC and p41 for erythema migrans (EM) and Lymphocytar Meningeoradikulitis (LMR) (Western Blot)

Diagnosis	reactive Proteins p41	pC	Ig Class	Number
EM	11	13	IgM	15[1])
LMR	13	10	IgM	20[1])
	14	3	1gG	15[2])

1) The sera were positive in the IgM-IFT-ABS test

2) The sera were positive in the IgG-IFT-ABS test

1.3 Immune response against p100, p41 and p17 (IgG Western Blot)

Diagnosis	p100	p41	p17	p100 or p41	p100 or p17	Number
ACA	8	8	9	10	10	10
Arthritis	11	12	8	12	11	12

1.4 Intrathecal Immune response with lymphocytar meningeoradiculitis (IgG Western Blot)

	local intrathecal immune response	Reactivity in serum	Number
p41	12	9	12
other proteins	7	12	12

Abb Table 2: Reactivity of Immune Sera (against various bacterial agents) with Proteins from B. burgdorferi (Western Blot).

Description of the Figures

Figures 1 a and b:

Reactivity of B. burgdorferi-infected Patients with Lysates of 5 Different B. burgdorferi Strains in the Western Blot Test.

The tested sera were from stages II and III (neuroborreliosis, stage II (IgM and IgG); acrodermatitis (IgG) and arthritis (IgG), stage III). The early immune response is directed against a narrow spectrum of borrelia proteins (pC and p41), independently of the test strain. The late immune response is directed against a broad range of borrelia proteins. The immune dominant proteins are (independently of the test strain) p100 (with variable molecular weight) and p41.

Figure 2

2a) Process Supervision (IgM-Western Blot) with Erythema Migrans

The small pC-protein can be the immune-dominant protein of the early immune response. Antibodies against p41 can arise later and be only weakly expressed. In longer-term illness, IgM-antibodies against p17 can also appear.

2b) IgG-Western Blot with Late Manifestations

IgG-antibodies recognize a broad spectrum of borrelia proteins. When using the strain PKo, the proteins p17 and p100 are

immune-dominant. p17 is strongly expressed from the strain PKo (in contrast to other strains; see Figure 1). The flagellin p41 was not recognized in two of these examples (Sera 1 and 2).

Figure 3

Pattern of DNA Amplification of the p41-coding Region.

A; Sector of B. burgdorferi-DNA with the p41 coding region (black line)

B; Magnification of the 5' or 3' end of the p41-gene with the corresponding DNA-sequences. Also shown is the translation start (ATG) as well as the stop-codon at the 3' end (TAA). The primer sequences used for the PCR are given under (primer 1) or over (primer 2) the p41 coding DNA double strand. A hybridization of the primers can take place only with the corresponding 3'-regions. The 5'-ends include non-hybridizing portions which establish the cleavage locations for restriction enzymes: GGATCC - BamHI; TCATGA - BspHI, at the 5'-end; GACGTC - PstI at the 3'-end.

Figure 4

Expression, Reactivity and Purification of Recombinant p41.

left portion: Coomassie-blue coloured SDS-polyacrylamide gel. The individual traces were activated as follows: 1, E.coli-lysate, negative control; 2, E.coli-lysate with pUC81y17 after IPTG-induction; the recombinantly produced p41 can be recognized as additional bands in the region around 45kDA; 3, the supernatant of the lysate from 2 after breaking up of the cells as described in

Example 4; 4, pellet-fraction of the lysated cells with the recombinant p41; 5, octyl-gluco-pyranoside supernatant; 6, as 5, but the pellet-fraction; 7-10, fractions after elutriation of p41 from a MonoQ-column using a salt gradient; traces 9 and 10 contain recombinant p41; depending upon the degradation evidence as well as incomplete translation, small fragments appear alongside the completed product, the fragments being however found also in authentic p41 material from B. burgdorferi.

right portion: immune-coloured Western Blot of an SDS-gel with tests of the Coomassie-coloured gel. The immune colouring was carried out with a monoclonal antibody as described in Example 6. Indicator of the traces or the tests such as Coomassie-coloured gel: trace 0, empty trace.

Figure 5

HPLC-elutriation Profile of p41 from an Ion Exchange Column with a Salt Gradient.

Following the anion exchange purification (MonoQ from Pharmacia) of p41, the antigen was return-dialysed on 4M were without salt, and again placed in the MonoQ-column in order to verify the purity. The elutriation profile shows only one protein adsorption spike. The smaller spike immediately in front of the main one corresponds to the p41-fragment, visible in Figure 4, trace 8, with a size of approximately 30kD (tested using Western Blot).

Figure 6:

IgG-ELISA with Recombinant p41 as Antigen.

The recombinant antigen purified in the anion exchanger (MonoQ) (see Figure 5) was applied in a concentration of 0.5 µg/ml. Seven sera from patients with a clinically defined lyme borreliosis and eight sera from healthy individuals were tested. Four sera of the lyme borreliosis patients were strongly reactive in the Western Blot with the recombinant p41 (= positive), 3 sera weakly reactive (= of marginal value), whereas the sera of the healthy individuals did not react (= negative). The IgG-ELISA showed a comparable result. Y-axis: optical density of the wave length of 486nm; marg. val = of marginal value.

Figure 7:

Reactivity of Monoclonal Antibodies Against Various B. burgdorferi Antigens.

Six monoclonal antibodies against B. burgdorferi were tested along with 30 different B. burgdorferi strains, four relapse fever borrelia strains and two different treponemas. In the Figure are shown three different B. burgdorferi isolates (1=B31, American strain; 2=PKo, German skin strain; 3=PBi, German liquor strain), a relapse fever borrelia (4=B. hermsii) and a treponema strain (5=T. phagedenis) as examples. The monoclonal antibody manufactured according to Example 6 was used.

Patent Claims

1. An immunologically active protein from Borrelia burgdorferi, characterized in that, it is present in a form free of other proteins deriving from Borrelia burgdorferi.
2. An immunologically active protein according to Claim 1, characterized in that it is created gene-technologically.
3. An immunologically active protein according to Claim 2, characterized in that it can be made with the use of DNA isolated from Borrelia burgdorferi.
4. An immunologically active protein according to Claim 3, characterized in that it can be made utilizing DNA isolated from Borrelia burgdorferi (DSM-No. 5662).
5. An immunologically active protein according to one of the preceding claims, characterized in that it has a molecular weight of approximately 41kDA.
6. An immunologically active protein according to Claim 5, characterized in that it shows the amino acid sequence

(insert from p.48 at top)

or partial sequences therefrom.

7. An immunologically active protein according to one of the Claims 1 through 4, characterized in that it has a molecular weight of approximately 22kDa.

8. An immunologically active protein according to Claim 1 or Claim 7, characterized in that it has the amino acid sequence

(insert from p.48 at bottom)

or a partial sequence therefrom.

9. An immunologically active protein according to one of Claims 1 through 4, characterized in that it has a molecular weight of approximately 17kDa.

10. An immunologically active protein according to one of Claims 1 through 4, characterized in that it has a molecular weight of approximately 100kDa.

11. An immunologically active protein according to Claim 1 or Claim 10, characterized in that it has the amino acid sequence

(insert from p.49)

or a partial sequence therefrom

12. An immunologically active protein according to one of Claims 1 through 4, characterized in that it has a molecular weight of approximately 31kDa.

13. An immunologically active protein according to Claim 1 or Claim 12, characterized in that it has the amino acid sequence

(insert from p.50)

or a partial sequence thereof.

14. A test kit for detecting antibodies against borrelia strains, characterized in that it includes at least one immunologically active protein in accordance with one of Claims 1 through 13, the said protein being capable of reacting with the antibodies present in the test liquid, and in that it includes at least one indicator component which makes it possible to detect complexes of immunologically active proteins and antibodies.

15. A test kit according to Claim 14, characterized in that it contains from 2 to 4 immunologically active proteins according to Claims 1 through 13.

16. A test kit according to one of Claims 14 or 15, characterized in that the indicator component is an antibody directed

against the antibody to be detected, the (indicator) antibody containing a marking.

17. A test kit according to Claim 16, characterized in that the marking consists of a radioactive isotope.

18. A test kit according to one of Claims 14 through 16, characterized in that the marking consists of an enzyme which can catalyse a colour reaction.

19. A test kit according to Claims 14 or 15, characterized in that the immunologically protein, or a monoclonal antibody directed against it, is biotinylized, and the indicator component is avidin or streptavidin with a covalently bound enzyme, particularly peroxydase.

20. A test kit according to one of Claims 14 through 16, characterized in that it is an ELISA test kit.

21. A test kit according to Claim 20, characterized in that at least one immunologically active protein according to one of Claims 1 through 13 is coupled to micro titer plates, and the indicator component consists of anti-human-immuno-globulin, especially IgG antibodies and/or IgM antibodies, to which an enzyme is coupled which catalyses a colour reaction.

22. The use of immunologically active proteins according to one of Claims 1 through 13 for the manufacture of vaccines for protection against infection by borrelia bacteria, preferably Borrelia burgdorferi strains.

Fig. 1: IgG Western Blot with 5 different strains as antigen

IgG and IgM response in stage II

IgG response in stage III

Neuroborreliosis, Stage II

Fig. 2a: IgM Western Blot for erythema migrans

Antigen: B. burgdorferi/Strain PKo

Patient A:	1	First examination
	2,3	Progress results after 1 and 3 weeks
Patient B:	1	First examination
	2-5	Progress results after 2, 9, 10 and 11 weeks

Fig. 2b: IgG-Western Blot for late manifestations

Antigen: B. burgdorferi/Strain PKo

1,2,3	acrodermatitis
4,5	arthritis

Fig. 3:

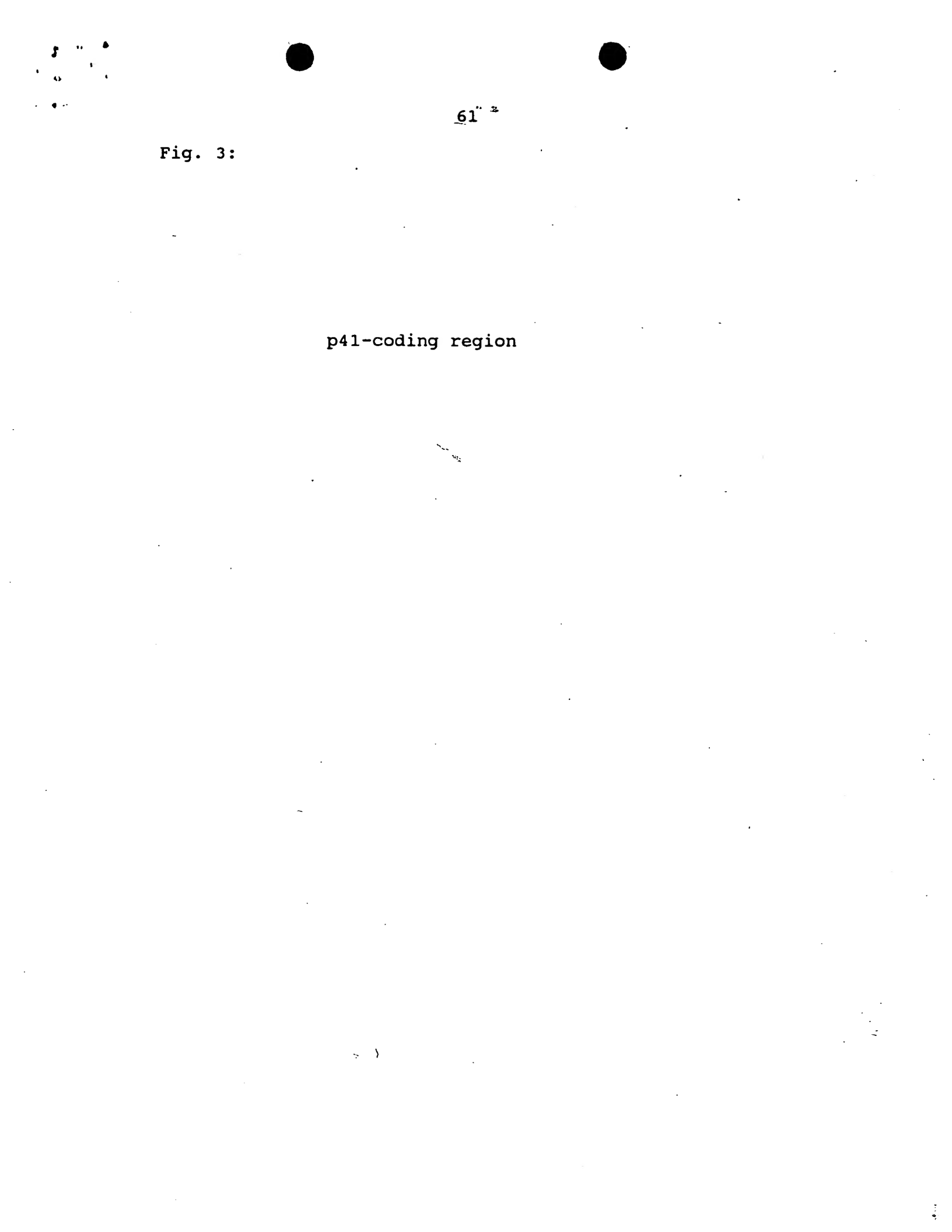

Fig. 4:

Fig. 5:

Elutriation Profile of p41 from an Ion Exchange Column

Fig. 6:

IgG - ELISA with recombinant p41 as antigen (0.5ug/ml)

positive marginal negative

Fig. 7a: Monoclonal antibodies against B. burgdorferi

Fig. 7b: Monoclonal antibodies against B. burgdorferi